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I, KAY WARD, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PP 7299 for a patent by BRESAGEN LIMITED filed on 24 November 1998.

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K Ward

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PROVISIONAL SPECIFICATION

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Invention Title: **Cryopreservation of Porcine Embryos and Methods for Producing Piglets Involving the Same.**

The invention is described in the following statement:

CRYOPRESERVATION OF PORCINE EMBRYOS AND METHODS FOR PRODUCING PIGLETS INVOLVING THE SAME

The present invention relates to methods for the cryopreservation of porcine embryos, particularly zona intact porcine embryos, and methods for producing live piglets from such embryos.

The production of live animals from frozen thawed embryos has been described for a number of species including cattle and sheep.

However the production of live piglets from frozen thawed embryos remains problematic. Only a small number of piglets have been produced using conventional techniques and all have been from perihatching embryos, and not zona intact porcine embryos. The perihatching embryo stage is unsuitable for most uses because the embryos are not surrounded by an intact zona pellucida and are subject to bacterial and viral infection. This is an all important requirement for the import/export of genetic material. Such protocols require embryos to be surrounded by an intact zona pellucida because it protects against bacterial and viral infection as mentioned above, thus reducing the risk of disease transmission.

The successful cryopreservation of porcine embryos remains largely illusionary with cryopreservation techniques used for cryopreservation of embryos from other species generally not being successful.

In one prior proposal, pig embryos have been subject to conditions which reduce the level of lipid in the embryo. In this proposal lipid was forced from the embryonic cells, resulting in a layer of lipid between the blastomeres and the zona pellucida. This lipid was moved by aspirating a lipid from the embryo using micro manipulation techniques. Removing lipids from embryos requires considerable technical skill, as well as much complexity.

The present invention seeks to overcome the problems associated with cryopreservation of zona intact piglet embryos, and seeks to provide simple, convenient and easily performed methods for the cryopreservation of zona intact porcine embryos, and for producing live piglets therefrom.

Summary of Invention

In accordance with a first aspect of this invention there is provided a method for the cryopreservation of porcine embryos, particularly zona intact porcine embryos, comprising centrifugation of embryos to polarise cytoplasmic lipid outside the embryonic cells, subjecting the embryos to low temperature conditions in the presence of a cryoprotectant which results in freezing of the embryo prior to lipid depolarisation, followed by low temperature storage of the frozen lipid polarised embryos.

Preferably, the embryos are vitrified by freezing in liquid nitrogen or other very cold fluid or gas which allows rapid temperature reduction.

In accordance with another aspect of this invention there is provided a method for producing piglets from zona intact embryos which comprises thawing a cryopreserved lipid polarised zona intact embryo and thereafter implanting the embryo into the uterus of a pregnancy competent female pig which at the conclusion of pregnancy term gives rise to live piglets.

Detailed Description of the Invention

The present invention provides for the cryopreservation of zona intact porcine embryos which hitherto have not been amenable to cryopreservation, and more particularly the successful production of piglets from the cryopreseved embryos.

The inventors have surprisingly found that centrifugation of porcine embryos, particularly zona intact porcine embryos, which polarises cytoplasmic lipid outside the embryonic cells, followed by exposure to low temperature conditions, preferably vitrification, in the presence of cryoprotectant, enables successful cryopreservation of the polarised embryos which

maintains their viability such that on implantation into the uterus of a pregnancy competent female pig, piglets can develop. Accordingly, in a first aspect of this invention there is provided a method for the cryopreservation of porcine embryos comprising centrifugation of embryos to polarise cytoplasmic lipid outside the embryonic cells, subjecting the embryos to low temperature conditions in he presence of a cryoprotectant which results in freezing of the embryo prior to lipid depolarisation, followed by low temperature storage of the frozen lipid polarised embryo.

Porcine embryos which may be subject to the methods of the present invention include zona intact embryos from the oocyte stage, through to late blastocysts, including morulae to mid-blastocysts stage, and hatched (non-zona intact) blastocysts.

Embryos may be prepared by standard techniques such as surgical recovery of zygotes, and embryos from within one to six days following mating. Alternatively, non-surgical collection of embryos is also possible. For example, embryos may be recovered at slaughter from fallopian tubes, or the uterine environment of slaughtered female pigs. The recovered embryos may be briefly cultured in a medium standardly used for embryo culture. Brief culturing or rinsing with standard embryo culture medium allows any blood or other components to be washed away from the embryos following removal from a donor animal. Whilst in no way essential, it is generally desirable to briefly culture the embryos prior to the methods of this invention.

The embryos are cultured in a cryoprotectant-containing solution prior to vitrification. The embryos need only be incubated in the cryoprotectant solution for a short period of time, for example from two minutes to one hour, more preferably from two minutes to 30 minutes, still more preferably from 3 minutes to 20 minutes.

Embryos may be incubated in a cryoprotectant-containing solution either prior to, during or after centrifugation, or both.

The cryoprotectant-containing solution in which embryos are incubated either prior to, during centrifugation, or after centrifugation, may contain any standard cryoprotectant established for use in the freezing of animal embryos, including glycerol, ethylene glycol, dimethylsulfoxide, propylene glycol and polyvinyl pyrrolidine, sucrose, trehalose, Ficoll, acetamide, egg yolk and the like. The concentration of cryoprotectant is used in an amount sufficient to replace to at least some extent water within the embryo, such that on rapid freezing ice crystal formation is prevented. By way of example, cryopreservatives may be present in an amount from 0.5M to 8M. One or more cryoprotectants may be used. The time in which embryos may be incubated in a cryoprotectant solution following centrifugation is insufficient to allow lipid repolarisation into the tissues of the embryo.

Embryos are centrifuged for a time sufficient to polarise cytoplasmic lipid from the embryonic cells to the outside of the embryonic cells, for example 1 to 15 minutes at 10,000 to 20,000g. The time period of centrifugation will depend upon the centrifugal force applied during centrifugation. At a centrifugal force of about 13000g polarisation takes place after about 8 minutes of centrifugation. It may be more convenient to centrifuge embryos in the presence of embryo culture medium, rather than in the presence of more viscous cryoprotective-containing solutions.

Embryo culture medium and cryoprotectant-containing solution for culturing embryo either before or after centrifugation may contain DNA synthesis inhibitors such as Cytochalasin B which relaxes cytoskeletal elements.

Following centrifugation to polarise lipid, the lipid polarised embryos are subject to low temperature conditions in the presence of a cryoprotectant which results in freezing of the embryo prior to lipid depolarisation. By lipid depolarisation is meant the return of lipid to the cells which was polarised outside the lipid cells by centrifugation. It is to be noted that on polarisation cytoplasmic lipid may be attached to cells but displaced outside the cells.

Low temperature conditions may be provided by slow cooling, rapid freezing and vitrification. In these techniques the embryo is frozen before the lipid returns to the cells (blastomeres). Vitrification may take place by placing the embryos in a vessel, and plunging the vessel into an extremely cold environment, such as liquid nitrogen or other liquefied and/or gaseous extremely cold substance. Alternatively, a vessel containing embryos may be rapidly frozen in an ultra-cold freezer, for example at temperatures below about -30°C. Any other apparatus or methods that enable rapid freezing may be used. In one example, embryos may be loaded into a straw which is heat sealed, and then plunged into liquid nitrogen. In another example, embryos may be pulled by capiliary action into a open pulled straw, which is then plunged into liquid nitrogen and subsequently stored (Vajta et al (1997) Cryoletters 18 191-5).

Embryos may be stored in a conventional freezer facility, at temperatures, for example, from -10°C to -70°C or more.

Frozen lipid polarised embryos may be thawed according to conventional embryo thawing techniques, such as incubation of the frozen straw at a temperature of 35°C to 39°C in a suitable culture medium. Thawed embryos may be washed in culture medium, further cultured briefly, and then introduced into the uterus of a pregnancy competent female pig. At the conclusion of pregnancy term the introduced embryos have developed to live piglets.

The present invention provides a simple and straight forward procedure for cryopreservation of porcine embryos, particularly zona intact frozen embryos On thawing and implantation of the embryos into the uterus of a pregnancy competent female pig, piglets may be produced in a manner which has not been achievable by the prior art.

Embryos may be subject to genetic manipulation prior to the process of this invention. In this regard one or more genes of interest may be inserted into the embryo by established techniques, such as using transfer vectors, homologous recombination and other established techniques for introducing genes into embryos. Alternatively, the embryos prior to

introduction into the uterus of a pregnancy competent female pig may be subject to genetic manipulation.

Certain embodiments of the present invention will now be exemplified with reference to the following non-limiting examples.

Example 1

Embryos were collected and washed thoroughly in culture medium (mPB1 - Quinn et al (1982) J. Reprod. Fert. 66:161-168) of 39°C three times.

The embryos, in the early blastocyst stage were cultured for 40 minutes in the standard embryo culture medium NCSU-23 with 10% foetal bovine serum (FBS) containing 7.5μg/ml Cytochalasin B at 39°C in a humidified environment of 5% CO_2 and air. After a five minute period of cooling from 39°C to 25°C, the embryos were cultured for 5 minuets in 6% BSA in BECM-3h, and then washed in 25% VS3a for approximtely three minutes (VS3a containing 6.5 m glycerol, 6% bovine serum albumin in BECM-3h (Dobrinsky et al (1996) Biol. Reprod. 55: 1069-1074). The embryos were centrifuged in a 1.5 Eppendorf tube (in the same media) at 13000g for about 12 minutes, recovered back into 25% VS3a, and then left in that media for a further five minutes. The embryos were then washed for 30 seconds in 65% VS3a, followed by a wash in 1ml VS3a before being loaded into a straw, heat sealing the straw, and plunging the embryos in the straw into liquid nitrogen.

In an alternative to storage in a heat-sealed straw, the embryos following a wash in the vitrification solution, are placed into a small drop of vitrification solution and drawn by capillary action into a narrowed hand pulled 0.25ml freezing straw (unsealed pulled straw, UPS). The straw is then plunged directly into liquid nitrogen.

Results of one experiment are set forth in Table 1.

TABLE 1

Freezing Method	Experimental Replicates	Embryo Stage	Embryo Number	Number viable (%) with blastocoels after culture for 24h	48h
Standard freezing method (BEVS)	2	Mor	4	0	0
	2	MBI	17	0	0
	2	Peri	13	2(15.4)	2(15.4)
BEVS/Sp	2	Mor	11	5(45.5)	6(54.6)
	3	MBI	43	27 (62.8)	26(60.5)
BEVS/UPS	3	Peri	17	10(58.8)	10(58.8)
BEVS/Sp/UPS	2	Mor	6	3(50)	5(83.3)
	5	MBI	44	33(75)	28(63.6)

TABLE 1
Survival of Porcine Embryos frozen at various stages, thawed and then cultured for 48h. The morphological stages examined were Mor, morulae, MBI, early to mid blastocysts; Peri, peri-hatching blastocysts. The treatment used were BEVS, Beltsville embryo vitrification system (Dobrinsky et al (1997) Theriogenology 47: 343); Sp, centrifuged, UPS, unsealed pulled straw.

Example 2

Method

Embryos were collected, washed throughly in mPB1 and then cultured for 35 minutes in NCSU-23 + 10% FBS with 7.5μg/ml Cytochalasin B at 39°C in an atmosphere of 5% CO_2 in air and 100% humidity. Morulae to middle blastocyst stages were centrifuged at 13000g for the last 10 minutes of this incubation in the culture medium containing the Cytochalasin B.

The embryos are then transferred to 2M ethylene glycol in mPB1 at 25°C for five minutes before being washed thoroughly in 8M ethylene glycol and 7% PVP in PBS and then placed into a small droplet of the vitrification media and loaded into an unsealed pulled glass by capillary action. The straw is then plunged into liquid nitrogen and stored.

Thawing is by placing the end of the straw containing the embryos into 1.2mls of 1M sucrose in PBS at 39°C. By blocking the open end with a finger, the fluid containing the embryos is forced out once, thawed, by the warming of the straw. Once collected, the embryos are placed into 1M ethylene glycol in mPBI for two minutes followed by 0.5M ethylene glycol in mPBI for a further 2 minutes, both at 25°C. Five minutes in mPB1 at 39°C completes the rehydration procedure. The embryos can then be prepared for culture or transfer.

TABLE 2

Results

Freezing Method	Embryo Stage	Replicates	Embryo Number	Number Viable (%) with blastocoels after culture for 24hrs	48hrs
E/UPS	MB1	1	5	0 (0)	0 (0)
	Peri	3	18	8(44.4)	6(33.3)
E/Sp/UPS	MB1	2	14	12 (85.7)	11(78.6)

TABLE 2
Survival of freshly collected porcine embryos, vitrified with 8m ethylene glycol and 7% PVP. Embryos stages were early to middle blastocysts (MBI) and peri-hatching blastocysts (Peri). Freezing methods were: E/UPS, vitrified in the medium described above in an unsealed pulled straw, and E/Sp/UPS, vitrified in a similar manner but centrifuged at 13000g during the last 10 minutes of the culture in NCSU-23 + Cytochalasin B (7.5μg/ml).

Transfer of Vitrified Embryos

The embryos once thawed using the appropriate technique are washed 3 times in mPB1 at 39°C before being held in mPB1 until just prior to transfer. They are then washed in media (PBS + 10% FBS + 2% Penicillin/Strepomycin solution (CSL: Penicillin G 5000μ/ml, Streptomycin sulphate 5000μg/ml)) at 39°C before loading into a Tomcat Catheter attached to a 1ml syringe followed by immediate transfer into one horn of the recipient animal.

TABLE 3

Experiment No.	Day of Transfer	Embryonic Stage	Embryo Number	Vitrification Technique	Results thus far
1	4	Early blastocysts	37	BEVS/Sp/UPS	Returned
2	4	Early blastocysts	32	BEVS/Sp/UPS	Returned
3	4	Peri-hatching (16 hatched)	37	BEVS/UPS	Pregnant

TABLE 3
Results of the transfer of porcine embryos, vitrified and thawed, into pseudopregnant recipients. Techniques used were: BEVS, Beltsville Embryo Vitrification System: Sp, centrifugation as described in methods: UPS, vitrified and stored in an unsealed pulled straw.

The two recipients from the Experiment 2 were confirmed as not pregnant 42 days after ovulation by progesterone assay. This was followed by a return to oestrus. The third recipient (Experiment 3) was confirmed as pregnant by progesterone assay 35 days after ovulation and by detection of a uterine artery pulse.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising" or the term "includes" or variations thereof, will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers. In this regard, in construing the claim scope, an embodiment where one or more features is added to any of claims is to be regarded as within the scope of the invention given that the essential features of the invention as claimed are included in such an embodiment.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for the cryopreservation of porcine embryos comprising centrifugation of embryos to polarise cytoplasmic lipid outside the embryonic cells, subjecting the embryos to low temperature conditions in the presence of a cryoprotectant which results in freezing of the embryos prior to lipid depolarisation, followed by low temperature storage of the frozen lipid polarised embryos.

2. A method according to claim 1 wherein the embryos are zona intact porcine embryos.

3. A method according to claim 1 wherein the embryos are vitrified in the presence of a solution containing one or more cryoprotectant agents.

4. A method according to claim 2 wherein the cryoprotectants are selected from dimethylsulfoxide, propylene glycol, ethylene glycol, glycerol, PVP, sucrose, trehalose, Ficoll, acetamide and egg yolk.

5. A method for producing live piglets from porcine embryos which comprises thawing a cryopreserved zona intact embryo produced according to claim 1 and thereafter introducing the embryo into the uterus or fallopian tubes of a pregnancy competent female pig which at the conclusion of pregnancy term gives rise to live piglets.

6. A method according to claim 3 wherein embryos are vitrified by freezing in liquid nitrogen.

7. A method according to claim 6 wherein embryos are frozen in a freezing vessel including cryologic vials or freezing straws including open pulled straws in which embryos are located by capillary action.

8. A method according to claim 2 wherein the embryos are in the morulae to mid-blastocyst stage.

5 DATED this 24th day of November 1998

BRESAGEN LIMITED

By Its Patent Attorneys

DAVIES COLLISON CAVE

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